Mutagenesis protocol for site direct mutagenesis

Introduction
This protocol in based in a routine Phusion® PCR by New England BioLabs
These guidelines cover a site direct mutagenesis.
Note the difference in the steps and program than a routine PCR.

Reaction Setup: We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity.

PCR recipe

Component	**20 µl Reaction**	**50 µl Reaction**	**Final Concentration**
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
Template DNA	100-500ng	100-500ng	variable
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling

PCR Program

STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	30 seconds
	98°C	5-10 seconds

16-20 Cycles*	55°C 72°C	10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	37°C**	1-2 Hrs***

*After complete the first half of the 8-10 cycles, stop the machine, let the reaction mix gradually cold, open the tube and add the following

** Set storage temperature at 37°C, another option is place the cooled tube inside a 37°C incubator.

Component	**20 µl Reaction**	**50 µl Reaction**	**Final Concentration**
10 µM Reverse Primer	1 µl	2.5 µl	<0.5 µM

After adding the primers, resume the remaining cycles.

***Once that the program is complete with the two primers, in the holding step add 1 µl DpnI restriction enzyme to the PCR tube directly. Only digestiong the PCR products for no more that 2 hrs. (Purification is not necessary at this stage).

Transform purified DNA into highly competent cells.

Screen the transformants for the desired mutation using colony PCR, restriction digest or sequencing as appropriate.

Extra
Primers phosphorylation of 5'

1. Mix:
2. 3µ 100µM sense oligo (final concentration 10µM)
3. 3µ 100µM anti-sense oligo (final concentration 10µM)
4. 3µ 10X T4 DNA ligase buffer
5. 2µ T4 Polynucleotide kinase (PNK)
6. 19µ double distilled water
   (Total volume 30 µL)

7. Incubate at 37^{o}C for 1.5Hrs
8. Heat kill PNK by 10 min at 70^{o}C